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Final Report for Award Number DAMD17-01-1-0485

Title of the Grant Proposal: Exploration of the regulation of breast cancer by the Angiotensin II receptor AT2

PI: Lakshmi Pulakat

Abstract:

Overexpression of ErbB2 and ErbB3 receptors, members of the ErbB receptor family, are correlated to the transformation of breast, ovarian and many other cell types into malignant tumors. This involvement of the ErbB receptor family in the control of cell proliferation and its role in human malignancies has led to intense studies of these receptors and their regulatory mechanisms. The ErbB family is consisted of four homologous members, the EGFR or ErbB1, ErbB2, ErbB3 and ErbB4, and these molecules can homodimerize or heterodimerize and activate growth-promoting signaling in response to binding of a number of structurally similar growth factors. This situation makes regulation of growth-promoting signaling by ErbB family receptors in cancer very complex. Identification of a mechanism to regulate growth-promoting cell signaling by these receptors may lead to the development of effective treatments to inhibit tumor growth in breast and ovarian cancer types that show ErbB2 and ErbB3 receptor overexpression. Our proposal was developed from our observation that the Angiotensin II (Ang II)receptor AT2 could interact directly with the ATP binding domain of the ErbB2 and ErbB3 receptors in yeast-two hybrid assay. We found that the 3rd ICL and C-terminal cytoplasmic domain of the AT2 are essential for this interaction. Since the 3rd ICL of the AT2 is involved in growth regulation and this region is needed for the interaction between the AT2 and the human ErbB3, we hypothesized that this interaction may result in growth regulation of the breast cancer cells that overexpress ErbB2 and ErbB3. We proposed that we will analyze whether expression of the AT2 in breast cancer cells that overexpress the ErbB2 and ErbB3 receptors such as MDA-MB-453, MDA-MB-175 and SUM-52PE will lead to:

a) growth arrest,
b) induction of apoptosis
c) induction of differentiation
d) reduction of phosphorylation of the ErbB2/ErbB3 receptors
e) reduction in the overexpression of the ErbB2/ErbB3 receptors

Our preliminary analysis using RT-PCR of the above breast cancer cell lines showed that none of them expressed native Angiotensin II receptors, although normal breast epithelial cells are known to express Ang II receptors. Therefore we focused on the MDA-MB-453 cell line. As mentioned in the annual report previously, our initial attempts to generate stable cell lines of MDA-MB-53 expressing the AT2 by cloning the

AT2 under the transcriptional control of the cytomegalovirus promoter present in the vector pCDNA were unsuccessful. We hypothesized that the AT2 expression could have caused inhibition of cell growth and that was why we were unable to isolate actively growing stable cell lines that express the AT2. As mentioned in the annual report, we then proceeded to generate vectors from which AT2 could be expressed in a regulated manner and we generated mammalian vectors expressing the AT2 and a mutant AT2 (in which the third intracellular loop of the AT2 is replaced with that of the AT1) by cloning these genes in pRevTRE (for regulated expression of the gene by tetracycline). By using the extended time, we also took another approach. We used transient transfection as a method to determine how AT2 expression affects the growth of this breast cancer cell line. Our results show that 48 hours post transfection we are able to co-immunoprecipitate the AT2 with ErbB2 suggesting that direct interaction between the AT2 and the ErbB2 exist in these cells. Exposure to Ang II was not necessary for this interaction. This was very interesting, since this observation implied that just expression of the AT2 is sufficient to regulate the ErbB2 mediated signaling. Similar experiments conducted with anti-erbB3 antibody also showed that the AT2 could co-immunoprecipitate with the ErbB3. We also found that nine days post transfection, the AT2-transfected cells showed significant reduction in the levels of ErbB3. This was determined by testing the protein levels of ErbB3 in the AT2-transfected cells and non-transfected cells by SDS-PAGE analysis of the cell lysates and Western blotting and probing with anti-ErbB3 antibody. This result showed that expression of the AT2 in these cells resulted in inhibition of overexpression of the ErbB3. Moreover, when ErbB2 was immunoprecipitated from the AT2 transfected cells and non-transfected cells 72 hours post-transfection and tested for phophorylation levels by subjecting the precipitated material to SDS-PAGE, Western blotting and probing with anti-phosphotyrosine antibody, we observed that the phosphorylation of the ErbB2 from the transfected cells was very low. Taken together these results imply that ;

a) AT2 interacts directly with the ErbB2 or ErbB3 receptors in breast cancer cells
b) Continuous AT2 expression inhibits the growth of breast cancer cells in a ligand-independent manner
c) AT2 expression results in inhibition of the phophorylation of the ErbB2 and ErbB3 receptors in these cells
d) AT2 expression also inhibits expression of the ErbB2 and the ErbB3 receptors and this situation could have played an important role in arresting the growth of AT2-expressing cells and making it difficult to obtain stable cell lines expressing the AT2.

In summary, the above results support our original hypothesis that the AT2 may inhibit the overexpression and phosphorylation of the ErbB 2 and ErbB3 and direct interaction exists between the 3rd ICL of the AT2 and the ATP binding domain of the ErbB2 or

ErbB3 receptors (these regions are identical in these receptors). Currently we are analyzing the effect of two AT2 mutants, the chimeric receptor in which the entire 3rd ICL of the AT2 is replaced with that of the AT1 and the M8, in which only nine amino acids in the 3rd ICL of the AT2 are replaced with that of the AT1. We are also in the process of preparing a manuscript describing these results and planning to submit a research proposal to Department of Army to investigate whether peptides corresponding to specific domains of the AT2 are capable of inhibiting the growth of breast cancer cells.

Introduction

The Angiotensin II receptor AT2 is a 363-amino acid protein and shares structural similarity and 34% homology at the amino acid level with the other Angiotensin II receptor AT1 [1, 2]. The exact physiological role of the AT2 is still emerging. Recently Vervoort *et.al.*, showed that lack of expression of the AT2 in brain, or mutations in the AT2 gene causes mental retardation in humans, and suggested that the AT2 has an important role in brain development and cognitive function [3]. AT2 is also known to play important roles in the development of kidney and urinary tract, and in the functions of ovary, uterus and pancreas [4-7]. The AT2 seems to function as a negative regulator of cell growth [8, 9]. In many cell lines the AT2 is known to induce apoptosis and this effect is mediated by the 3rd ICL of the AT2 [10-12]. The aim of this research proposal is to investigate whether the overexpression and activation of the Angiotensin II receptor AT2 causes inhibition of cell growth and promotion of cell differentiation in breast cancer cells. This research stems from our previous studies using yeast two-hybrid assay which showed that the growth-inhibiting AT2 interacts directly with the cancer-promoting ErbB2/ErbB3 receptors. Specifically, these studies showed that the 3rd intracellular loop and the C-terminal cytoplasmic domain of the AT2 are needed for its interaction with the ATP-binding domain of the ErbB2/ErbB3 receptors. Since the domains of the ErbB2/ErbB3 receptors that interact with the AT2 receptor are located within the 21 amino acids that separate the residues GlyXGlyXXGly, and Lys (the residues predicted to be important for ATP binding), it is reasonable to assume that an interaction between the AT2 receptor and the ErbB2/ErbB3 receptors may result in influencing the ATP binding properties of the ErbB2/ErbB3 receptors. This situation may affect the tyrosine kinase activity of the ErbB2/ErbB3 receptors and the ErbB2/ErbB3 receptor-mediated cell proliferation. Moreover, since the AT2 receptor is known to inhibit cell proliferation and promote differentiation in many different cell types, the interaction between the ATP binding domain of the ErbB2/ErbB3 receptors and the 3rdICL and the c-terminal of the AT2 may also cause differentiation of these

cancer cells. Thus, the research proposed in this grant proposal is directed to initiate experiments to test these possibilities.

Key Research Accomplishments:

1) As mentioned in the proposal, expression of angiotensin II receptors in three breast cancer cell lines, MDA-MB-453, MDA-MB-175 and SUM52-PE were tested by RT-PCR analysis using appropriate primers and total RNA isolated from these cell lines. No band corresponding to the AT1 or the AT2 were obtained when the products of RT-PCR were subjected to electrophoresis on 0.8% agarose gels. To further test this idea, we tested the total cell lysate prepared from MDA-MB-453 for the presence fo the AT1 and AT2 proteins by subjecting the lysate to SDS-PAGE, Western blotting and probing with either the AT1 or the AT2 antibody. Again no band corresponding to the AT1 or the AT2 were visible in the autoradiograms. Another breast cancer cell line, MCF-7 that expresses the AT1, served as the positive control in the experiment to test for the presence of AT1 in MDA-MB-453 cells. AT2-transfected MDA-MB-453 cells served as the positive control in the experiments to test whether the non-transfected MDA-MB-453 cells expressed any native AT2 receptors. Ligand-binding experiments using ^{125}-I labeled Ang II also showed no binding to MDA-MB-453 cells indicating that these cells did not express any native Angiotensin II receptors. Taken together these results lead us to conclude that none of the breast cancer cell lines tested could express either the AT1 or the AT2 receptors of Angiotensin II.
2) SDS-PAGE analysis, Western blotting and probing with anti-ErbB2 or anti-ErbB3 antibodies of the lysates of the MDA-MB-453 cell line showed detectable levels of ErbB2 and ErbB3 proteins. Next, the ErbB2 and ErbB3 were immunoprecipitated from these cell lysates and their phosphorylation status was tested by anti-phosphotyrosine antibody. It was found that anti-phosphotyrosine antibody could bind both proteins suggesting that they were phosphorylated. Thus, the MDA-MB-453 cells express high levels of the ErbB2 and ErbB3 and these proteins remain constitutively phosphorylated in these cells even in the absence of any ligand binding. These observations are consistant with previous reports regarding the expression and phosphorylation status of these two proteins in this breast cancer cell line.
3) Expression of the AT2 in MDA-MB-453 cells for long periods of time (about a month) results in extremely reduced growth and cell death. When the cells are collected 48 hour post transfection, cells are still healthy and they still express the ErbB2 and the ErbB3 proteins as determined by SDS-PAGE analysis, Western blotting and probing with anti-ErbB2 or anti-ErbB3 antibodies of the lysates of the AT2-transfected MDA-MB-453 cells.

4) When the the ErbB2 or the ErbB3 are immunoprecipitated from the AT2-transfected cells 48 hours post transfection, and the immunoprecipitated pellet was subjected to SDS-PAGE analysis, Western blotting and probing with anti-AT2 antibody, it was found that the AT2 was co-immunoprecipitated with the ErbB2 or the ErbB3. This observation implies that direct interaction between the AT2 and the ErbB2/ErbB3 receptors exist in mammalian cells even in the absence of Ang II binding to the AT2.
5) When the ErbB2 was immunoprecipitated from the AT2-transfected cells 72 hours post transfection and after exposure to Ang II (100nM for 2 hours), it was found that the levels of ErbB2 that was immunoprecipitated was significantly low when compared to that from the AT2-transfected cells that were not exposed to Ang II or exposed to the AT2 antagonist PD123319. This result showed that exposure to Ang II causes a sudden decrease in the ErbB2 expression levles in the MDA-MB-453 cells that express the AT2.
6) Analysis of the levles of ErbB2 and ErbB3 proteins in the AT2-transfected MDA-MB-453 cells nine days post transfection showed highly reduced expression of the ErbB2 and the ErbB3 . This experiment was carried out by subjecting the lysates of these cells to SDS-PAGE analysis, Western blotting and probing with anti-ErbB2 or anti-ErbB3 antibodies. This result implied that continuous expression of the AT2 in these cells could cause inhibition of the expression of the ErbB2 and the ErbB3 receptors. Taken together these results suggest that exposure to Ang II can speed up the reduction of expression of the ErbB family receptors in MDA-MB-453 cells, however, prolonged expression of the AT2 could also bring about the same result in the absence of Ang II.
7) When the ErbB2 or ErbB3 was immunoprecipitated from the above AT2-transfected cells and the phosphorylation status of these proteins was analyzed by subjecting the immunoprecipitated pellet for SDS-PAGE analysis, Western blotting and probing with anti-phosphotyrosine antibody, it was found that there was negligible phosphorylation. This observation implied that expression of the AT2 causes reduction in the phosphorylation levles of the ErbB2 or ErbB3 receptors in MDA-MB-453 cells.
8) A pCDNA construct carrying the chimeric receptor (the AT2 receptor in which the 3rd ICL of the AT2 was replaced with that of the AT1) and another pCDNA construct carrying the M8 mutant of the AT2 (the AT2 receptor in which only nine amino acids in the 3rd ICL were replaced with that of the AT1 were also introduced into the MDA-MB-453 cells. Currently experiments are in progress to test whether exposure to Ang II has any effect on the ability of the chimeric receptor to co-immunoprecipitate with the ErbB2.
9) A pCDNA construct carrying the AT1B gene was also introduced into the MDA-MB-453 cells. Immunoprecipitation of the AT1B using anti-AT1-antibody and

analysis of the pellet for co-immunoprecipitation of the ErbB2 or the ErbB3 by SDS-PAGE analysis, Western blotting and probing with anti-ErbB2 or anti-ErbB3 antibodies showed that neither ErbB2 nor ErbB3 could co-immunoprecipitate with the AT1B. This result implied that the AT1 receptor may not interact with the ErbB2 or the ErbB3 in a similar manner to that of the AT2.

Reportable Outcomes

1) A manuscript entitled "Regulation of the ErbB2 and ErbB3 receptor signaling in human breast cancer cell line MDA-MB-453 by the AT2 through direct protein-protein interaction" by Lakshmi Pulakat, Chirag Mandavya, Sucharitha Balasubramaniam and Nara Gavini is currently under preparation for submission to Journal of Biological Chemistry.
2) Lakshmi Pulakat and Nara Gavini (2002) Structure-Function of the Angiotensin II Receptor AT2 and Signaling in Breast Cancer. Abstract published in "Era of Hope Proceedings. Volume 1 and pp P3-25 A poster describing this work was presented during the 'Era of Hope' Meeting at Orlando, Fl., on Sept 25-29, 2002.
3) Dieter Knowle, a Ph.D. student who characterized the interaction between the rat AT2 and the human ErbB3 received his Ph.D. degree on August 2001. His Dissertation was chosen as the "Distinguished Dissertation of 2001" by Bowling Green State University in November 2001.
4) PI (Dr. Pulakat) was awarded "Certificate of Appreciation" by the Distinguished Dissertation Award Committee of Bowling Green State University for her guidance of Distinguished Dissertation Award Recipient, Dr. Dieter Knowle, November 2001.

Conclusions

Our studies have elucidated a new signaling mechanism for the AT2 by which this receptor could exert its growth regulatory effects. The idea that the AT2 could directly interact with the ErbB2 and the ErbB3 receptors came from our original observation using yeast two-hybrid assay. However the critical question that remained was that whether such interaction between a seven transmembrane domain protein such as the AT2 and a one-transmembrane domain protein such as the ErbB3 could actually occur in mammalian cells. Our results generated from this study conclusively showed that the AT2 could interact with both ErbB2 and ErbB3 receptors in breast cancer cell line MDA-MB-453 even in the absence of its ligand, Ang II. Moreover our results also showed that expression of the AT2 alone is enough to inhibit cell growth of MDA-MB-453 cells and to reduce/inhibit the expression and phosphorylation of the ErbB2/ErbB3 receptors in this breast cancer cell line. It seems that prolonged expression of the AT2 can regulate the overexpression and phosphorylation of the ErbB2/ErbB3 receptors,

however, exposure of AT2-expressing MDA-MB-453 cells to Ang II enhances this inhibitory effect of the AT2. In summary, the financial support provided by the 'Concept Award' has helped us to elucidate the nature of regulation exerted by the AT2 receptor on cells overexpressing the ErbB2/ErbB3 receptors and also gain invaluable insights into the possible role time-dependent expression of the AT2 (even in the absence of ligand) in changing the fate of cells that highly express the ErbB2 and ErbB3 receptors (such as Schwann cells) during development. We are now in the process of preparing a manuscript describing these results to submit to Journal of Biological Chemistry. We have published an abstract describing these results in the 'Era of Hope Proceedings' (Volume 1 and pp P3-25) and also presented our results in the form of a poster in the meeting held at Orlando, Fl., on September 25-28, 2002. Dieter Knowle, a Ph.D. student in the PI's lab who characterized the interaction between the AT2 and the human ErbB3 received his Ph.D. degree in August 2001 and is currently working as a Post-Doctoral Fellow at Medical College of Ohio. Toledo. His dissertation received the "Distinguished Dissertation Award" for the year 2001 by Bowling Green State University and the PI received the "Certificate of Appreciation" by the Distinguished Dissertation Award Committee of Bowling Green State University for her guidance of Distinguished Dissertation Award Recipient, Dr. Dieter Knowle, November 2001. Two new graduate students, Chirag Mandavya and Sucharitha Balasubramaniam, are currently working on this project and are co-authors in the manuscript that is being prepared based on the results from this project for submission to J. Biol. Chem. As evident from the above description, the extended time had helped us to achieve the maximum benefit from the Concept Award, and provided us with valuable results to support our proposal to be submitted to the Department of Army to continue this research to determine whether AT2 peptides can regulate cell growth in breast cancer.

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